Diffusion Cannot Govern the Discharge of Neurotransmitter in Fast Synapses

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ABSTRACT In the present work we show that diffusion cannot provide the observed fast discharge of neurotransmitter from a synaptic vesicle during neurotransmitter release, mainly because it is not sufficiently rapid nor is it sufficiently temperature-dependent. Modeling the discharge from the vesicle into the cleft as a continuous point source, we have determined that discharge should occur in 50–75 μs, to provide the observed high concentrations of transmitter at the critical zone.

INTRODUCTION

The process of neurotransmitter release from nerve terminals has been intensively investigated for over four decades. Nevertheless, surprisingly little is known about the actual molecular and biophysical mechanisms linking exocytosis with the arrival of an action potential at the terminal.

The salient aspects of the sequence of events known to take place in a chemical synapse are the following. An action potential propagating along the axon reaches the nerve terminal and depolarizes the presynaptic membrane, which in turn increases membrane conductivity to Ca^{2+}. As a result, calcium ions flow into the terminal thereby increasing the intracellular calcium concentration. According to some investigators, in parallel and independently of calcium influx, the depolarization also activates the release machinery, rendering it sensitive to Ca^{2+}. This is called the Ca^{2+} voltage theory (Parnas et al., 1990). Activation of the release machinery, perhaps together with intracellular calcium, triggers exocytosis of the vesicle containing the neurotransmitter. The released neurotransmitter traverses the synaptic gap and generates the postsynaptic current.

Irrespective of the molecular mechanisms governing various steps of neurotransmitter release, the last step must be discharge of the vesicle contents into the synaptic cleft. Neither in fast nor in slow systems is there a comprehensive theory to account for the process of discharge. For fast synapses, the common view is that discharge occurs by means of diffusion (e.g., Almers et al., 1991; Klein et al., 1982).

In the present work we address the specific question of whether diffusion can be the mechanism underlying discharge in fast synapses. Using both pre- and postsynaptic considerations, we rule out the possibility of discharge via diffusion, mainly because it is not sufficiently rapid nor sufficiently temperature-dependent.

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PRESYNAPTIC CONSIDERATIONS

There are several experimental findings, using presynaptic data, that could help in understanding the nature of the process of discharge in fast releasing systems.

1. After an applied pulse, there is always a period of time during which no release is seen. The origin of this minimal delay reflects the events that occur in the presynaptic terminal after the depolarizing pulse. More precisely, the minimal delay is composed of the minimal time necessary for the chemical reactions promoting release to take place, together with the duration of the vesicle discharge. At room temperature (20°C) in different neuromuscular junctions the minimal delay can be very short, in the range of 0.5–0.6 ms (e.g., Katz and Miledi, 1965a; Datyner and Gage, 1980; Parnas et al., 1989). In the squid giant synapse the minimal delay is even less, about 0.2 ms (Llinas et al., 1982). Thus, the duration of the vesicle discharge in the neuromuscular junction can be at most 0.5 ms at room temperature but presumably much less. By comparison, in chromaffin cells the discharge from a single granule was shown to last tens of milliseconds (Chow et al., 1992).

2. The minimal delay is very sensitive to temperature. It is shorter at higher temperatures, with Q_{10} as high as 3–4 (Katz and Miledi, 1965b; Barrett and Stevens, 1972; Dudel, 1984; Parnas et al., 1989). Temperature dependence in the fast steps would not be observable. Thus, the time-limiting step in the sequence of presynaptic events should exhibit marked temperature dependence.

3. During the early stages of exocytosis, vesicles from neurons and other secretory cells appear to be connected to the extracellular space by narrow pores. By measuring the conductance of the fusion pore of the mast cell, Spruce et al. (1990) found that the pore must have molecular dimensions. Having assumed that the pore has a cylindrical form, they estimated its length to be 10–15 nm (two membrane thicknesses), and its diameter to be about 2 nm. After the pore opens, its diameter increases with the median rate of 0.8 nm/ms (Spruce et al., 1990).

To confront the experimental findings concerning minimal delay and temperature, we will calculate the time of diffusive discharge through a suitable pore.

DISCHARGE OF A QUANTUM VIA HINDERED DIFFUSION

Measurements of the diameter of the synaptic pore cannot be obtained with existing technology. Hypothesizing that the biophysical mechanisms underlying membrane fusion and pore expansion from synaptic vesicles and secretory granules have common features, we assume that the synaptic pores are similar in size to those in mast cells. This implies that at the end of the discharge (<0.5 ms) the pore diameter will be at most 2.4 nm.

The size of neurotransmitter molecules, such as acetylcholine (ACh), is comparable to the radius of the synaptic pore. When stretched out in its *all-trans* configuration, an ACh molecule is ~1 nm long and ~0.2 nm in width (Chotia and Pauling, 1968). Thus, the most adequate way to describe the diffusive discharge through the fusion pore is by considering hindered diffusion, wherein the actual diffusivity of molecules in the narrow pore is lower than that in an unbounded fluid.

The value of the hindered diffusion coefficient of nonspherical molecules, which most transmitters are, lies between the value of hindered diffusivity for spherical molecules with a given Stokes-Einstein radius, $r_s = kT/6\pi\eta D_\infty$, and the diffusion coefficient in an unbounded fluid, D_∞. (T is the absolute temperature and η is the viscosity.) From the calculated diffusion coefficient of ACh in the cleft, $4 \cdot 10^{-6}$ cm^2/s (Land et al., 1980), the Stokes-Einstein radius of ACh at room temperature is of the order 0.5 nm. Following Deen (1987), the hindered diffusion coefficient is $D = K_d D_\infty$, where K_d is the enhanced drag acting on a single molecule. The coefficient K_d depends on the ratio λ between the solute radius and the pore radius ($\lambda = r/r_s$). For the pore expanding during 1 ms with the given rate $b = 0.4$ nm/ms, the average pore radius is $r = 1.2$ nm and $\lambda \sim 0.4$. Using the formula for enhanced drag given by Deen (1987), $K_d \sim 0.28$. Consequently, a lower bound for the diffusivity of ACh molecules in a pore of radius 1.2 nm, is about one fourth of their diffusivity in the cleft, $D \sim 10^{-6}$ cm^2/s.

Diffusion of solute from a limited volume through a pore can be described according to Fick's law

$$\frac{\partial n}{\partial t} = -DS\frac{\partial C}{\partial x}. \qquad (1)$$

Here x is the coordinate along the pore length, $n(t)$ is the number of solute molecules at time t in the vesicle, $C(t, x)$ is the solute concentration at time t and position x in the vesicle, $S(t)$ is the cross-section of the expanding pore, D is the diffusion coefficient of solute in the vesicle and the pore, and $-DS \cdot \partial C/\partial x$ is the efflux from the vesicle (Fig. 1).

We will make several assumptions concerning Eq. 1. With the diffusion coefficient of ACh, 10^{-6} cm^2/s, reequilibration of the solute inside the vesicle occurs rapidly: $R^2/4D \sim 1$ μs. Thus, the concentration in the vesicle can be regarded as uniform, so that $C(t) = n(t)/V$. A virtually constant gradient in the pore is also established rapidly, since $L^2/4D \sim 0.2\,5$ μs. Hence, $\partial C/\partial x \sim (C(t) - C^e)/L$. C^e, the concentration at the pore exit, i.e., in the cleft, is assumed to be negligibly small and is fixed at 0. This assumption is obviously wrong but for non-zero extravesicular concentrations diffusive discharge is slower (see section on sensitivity of conclusions), so that our estimates will be conservative.

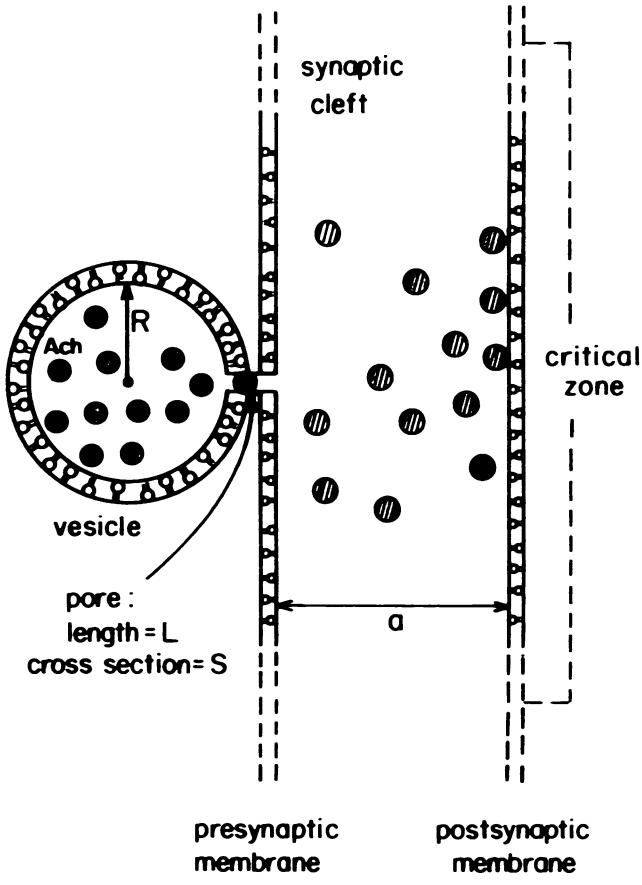

FIGURE 1 Schematic view (approximately to scale, except for molecules) of the synaptic vesicle connected to the plasma membrane (bilayer character not fully indicated) by a narrow pore and emptying its contents primarily onto the critical zone at the postsynaptic membrane. Typical values: internal vesicle radius from the neuromuscular junction, R = 185 Å (Heuser and Reese, 1973); pore length, L = 100 Å and initial pore radius, r = 10 Å (Spruce et al., 1990); cleft height, a = 500 Å.

Another parameter that could be of importance is the change in vesicle volume owing to the water flow through the permeable membrane. In the Appendix we estimate that volume changes are very small (~2%); we thus regard V as a constant.

The next important parameter is the cross-section of the expanding pore, $S(t)$. A reasonable approximation is that the pore radius grows linearly with time: $r = r_0 + bt$ (see Fig. 2 *D* in Spruce et al., 1990). Here r_0 is the initial pore radius and b is the rate of the radius expansion. If all the above assumptions are incorporated into Eq. 1, diffusive emptying is described by

$$\frac{dC}{dt} = -\frac{D\pi(r_0 + bt)^2 C}{VL}, \qquad C|_{t=0} = C_0. \qquad (2)$$

The solution for Eq. 2 is

$$C = C_0 \exp\left\{ -\left(t + \frac{b}{r_0} t^2 + \frac{b^2 t^3}{r_0^2 3} \right) \Big/ \tau \right\}, \qquad \tau = \frac{VL}{\pi r_0^2 D}. \tag{3}$$

Using Eq. 3, one can evaluate the mean time that is required for the diffusion of a certain amount of substance through the pore of changing radius. When $b = 0$ (no expansion), Eq. 3 reduces to an equation previously presented by Almers et al. (1991), who stressed that use of this equation requires that transmitter must be free, as it is the case in clear synaptic vesicles.

In Fig. 2 we present graphs for the concentration ratio $C(t)/C_0$ calculated using Eq. 3 for fixed and expanding pore sizes and for two diffusion coefficients. The upper solid line represents the dimensionless concentration in the vesicle as a function of time for the hindered diffusion through an expanding pore. The two dashed lines are for fixed pore radii: the initial (a) and average values (c), respectively. During the time of the minimal delay (0.5 ms) about 50% of the initial vesicle contents are released.

To justify our result, obtained from the approximate Eq. 2, a number of computer simulations of three-dimensional diffusion were performed using the computer program FIDAP (Engelman, 1991). We considered a sphere connected to a cylinder, which represent the vesicle and the pore. Initially neurotransmitter molecules were assumed to be present only in the sphere. The concentration at the pore exit was assumed to be 0, giving an upper bound on the discharge times. The discharge time was determined by monitoring the average concentration of the solute still remaining in the vesicle. The approximate Eq. 3 in fact gives a good approximation for the time of discharge. The numerical solution is given by asterisks in Fig. 2.

In assessing the significance of the results in Fig. 2, we note that there is considerable evidence that exocytosis results in the discharge of all, or almost all, of the vesicle contents (Kuffler and Yoshikami, 1975; Wagner et al., 1978; Stevens, 1993; Kandel and Schwartz, 1981). If for definiteness, we assume that discharge expels 95% of the initial

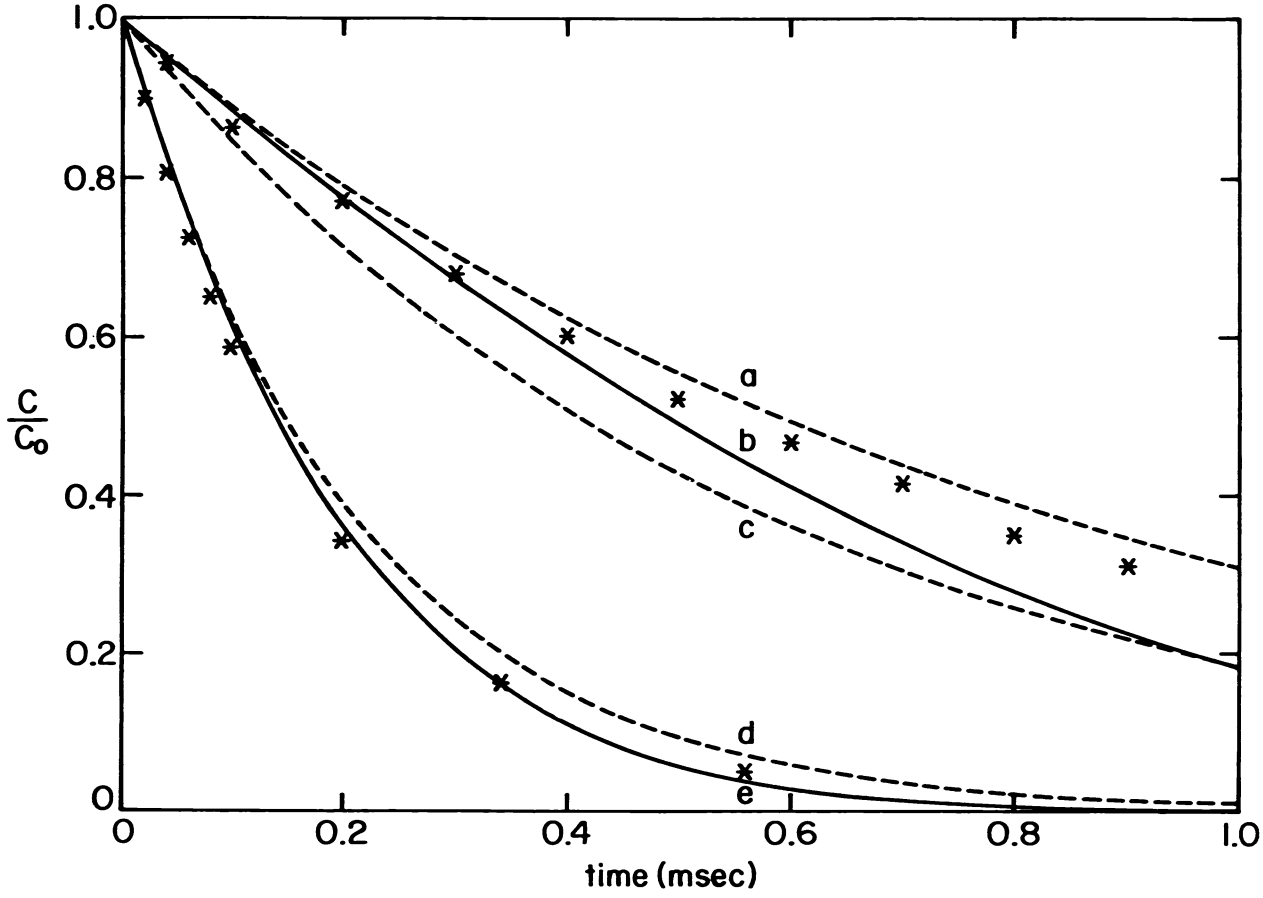

FIGURE 2 Ratio of the average concentration in the vesicle, C, and the initial concentration, C_0, as a function of time ($t \leq 1$ ms). Solid lines (b) and (e) represent results from the approximate formula (Eq. 3) for hindered and ordinary diffusion respectively; dashed lines represent results for the fixed pore radii: (a) hindered diffusion through initial pore, (c) hindered diffusion through average pore, (d) ordinary diffusion through initial pore. Vesicle and pore parameters are the same as in Fig. 1; average pore radius, $r = 1.2$ nm; $D_h = 10^{-6}$ cm^2/s, $D_\infty = 4 \cdot 10^{-6}$ cm^2/s; rate of pore radius expansion, $b = 0.4$ nm/ms (Spruce et al., 1990).

vesicular contents we find that with hindered diffusivity and with the parameters of the vesicle and the pore discussed above (Figs. 1 and 2), the duration of the vesicle discharge would be ~1.5 ms (Eq. 3). This value is much longer than the upper limit, the observed time of minimal delay.

Our calculations show that the discharge cannot be governed by hindered diffusion because it is too slow. Doubt can be cast on our results because we used the hindered coefficient for spherical molecules instead of the higher value of the elongated transmitter molecules. If were possible to obtain a more accurate hindered diffusion coefficient, the calculated discharge time might possibly be somewhat smaller. On the other hand, two assumptions acted in the opposite direction: we underestimated the discharge time by neglecting transmitter concentration in the cleft (see section on sensitivity of conclusions) and by taking the average pore radius when evaluating the hindered diffusion coefficient. The various approximations might roughly cancel. In any case, it is unlikely that the effect of the approximations will change our result that diffusive discharge is several times too slow.

DISCHARGE OF A QUANTUM VIA ORDINARY DIFFUSION

Hindered diffusion is the appropriate mechanism of diffusion of large molecules in the narrow pore. Nonetheless, we will estimate an upper bound for the duration of discharge by considering ordinary diffusion. As we will see, this gives an independent way to rule out diffusion. According to Eq. 3, with the diffusion coefficient of ACh in the cleft of $4 \cdot 10^{-6}$ cm^2/s, emptying of 95% of the vesicle contents through the expanding pore occurs in ~0.53 ms (Fig. 2). Hence, even if we take the highest reported diffusion coefficient, the diffusive discharge would still be longer than the minimal delay of about 0.5–0.6 ms at room temperature, or would be a time-limiting step among all the presynaptic events. However, according to the Einstein formula, diffusion exhibits only linear dependence on temperature and therefore cannot account for the observed high temperature dependence of the minimal delay. For shorter minimal delays (0.2 ms) even the fastest diffusive discharge is too slow.

POSTSYNAPTIC CONSIDERATIONS

We have used presynaptic data to suggest that the discharge of the vesicle contents is not likely to be achieved by diffusion. In this section we will strengthen our conclusions by considering postsynaptic data.

A quantum of neurotransmitter liberated from a vesicle moves across the synaptic cleft to the postsynaptic membrane, where it spreads only over a small critical area opposite the point of release and binds to highly concentrated receptors (Land et al., 1980) (Fig. 1). The critical area was estimated to be ~0.3 μm^2 at the frog end-plate. ACh attains high concentration throughout the critical area, with an average value during quantal response of $\sim 10^{-3}$ M (Matthews-Bellinger and Salpeter, 1978). According to other data, for a critical area of 0.2 μm^2 the average concentration is $3 \cdot 10^{-3}$ M (Fertuck and Salpeter, 1976), or even $5 \cdot 10^{-3}$ M (Land et al., 1980).

The forward rate constant for transmitter binding to receptors is typically 10^8 M^{-1} s^{-1}. With concentrations of magnitude 10^{-3} M throughout the critical area, the fastest effective time constant of binding will be roughly 10 μs. If hydrolysis is taken into account this time will be increased. Consistent with the postsynaptic potential rise time of tens of microseconds, this means that throughout the critical area concentrations of $\sim 10^{-3}$ M must persist for at least 10 μs.

Many authors (e.g., Land et al., 1984; Wathey et al., 1979) assumed that in order to model high concentrations at the critical zone, a quantum of ACh instantaneously appears in the cleft. Our calculations using Eq. 4 confirmed that in the case of instantaneous discharge diffusion of transmitter across the cleft and throughout the critical zone can indeed provide the estimated concentration. In reality, transmitter discharge from the vesicle of course takes a finite time. Molecules have to traverse the pore region filled with viscous fluid to leave the vesicle and appear in the cleft. We will now calculate the maximal permissible time of the discharge to provide the high observed concentration.

To do so, we employ an idealization of the vesicle discharge into the cleft as a point source situated on a "presynaptic plane" in a region bounded by two impermeable planes, representing the pre- and postsynaptic membranes. The distance between the planes is determined by the height of the cleft. The notion of a point source is justified because the pore radius through which transmitter molecules are liberated, ~0.001 μm, is negligibly small in comparison with the radius of the critical area, $R = 0.3$ μm (Matthews-Bellinger and Salpeter, 1978).

If at $t = 0$ a quantum, $Q = 10^4$ molecules (Kuffler and Yoshikami, 1975), is instantaneously discharged from the point source, the concentration at the postsynaptic membrane is given by

$$C(R, t) \sim \frac{Q}{4\pi a D t} e^{-R^2/4Dt}, \tag{4}$$

(Carslaw and Jaeger, 1962). Let the point source liberate transmitter during time θ at a constant rate q, where $q\theta = Q$. Integrating Eq. 4 over the duration of release we obtain the relevant solution

$$(R, t) \sim \frac{q}{4\pi a D} \int_0^{\theta} e^{-R^2/4D(t-t')} \frac{dt'}{t - t'}. \tag{5}$$

Fig. 3 shows the distribution of neurotransmitter at the critical area from a source of a duration 50 μs. One can see that for a such brief discharge the concentration is sufficiently high (10^{-3} M) and is already rather uniform 10 μs after the end of discharge. At 25 μs the concentration is even more uniform and is still sufficiently high. For a duration of 100 μs, the concentration of 10^{-3} M is attained only in the center

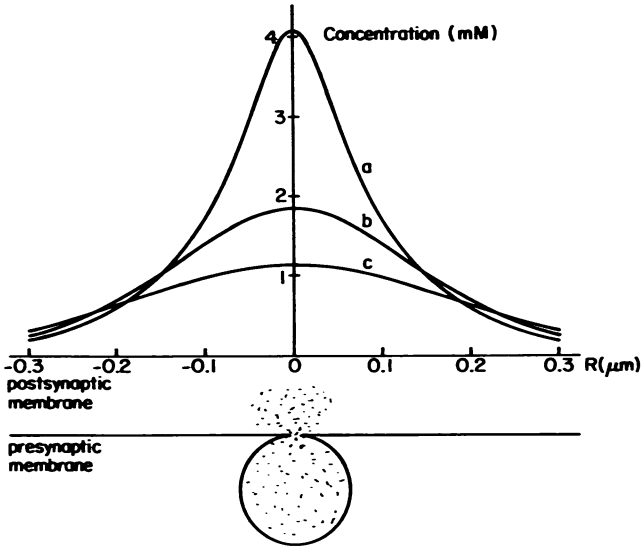

FIGURE 3 Continuous point source of transmitter of duration $\theta = 50$ μs. Transmitter concentration throughout the critical area at three values of time: (*a*) $t = 1$ μs after the end of discharge (~1 μs is time to diffuse across the cleft); (*b*) $t = 10$ μs; (*c*) $t = 25$ μs. See Eq. 5.

of the critical area, while there is a steep gradient across the critical zone (Fig. 4). After 25 μs, the concentration is much lower than required.

We conclude that a discharge of 100 μs in duration is too long to account for the postsynaptic data mentioned above (Matthews-Bellinger and Salpeter, 1978; Fertuck and Salpeter, 1976; Land et al., 1980). The optimal durations are 50–75 μs or less. This puts the diffusive discharge out of consideration even for the highest possible diffusion coefficient of small molecules in water (10^{-5} cm^2/s).

SENSITIVITY OF CONCLUSIONS

In this section we shall check the sensitivity of our conclusions to key parameter values, the magnitude of the diffusion coefficient and the width of the pore. Before doing this, however, we will examine the effect of an approximation that we know to be somewhat inaccurate (albeit conservative), namely the assumption that the concentration is 0 at the pore mouth.

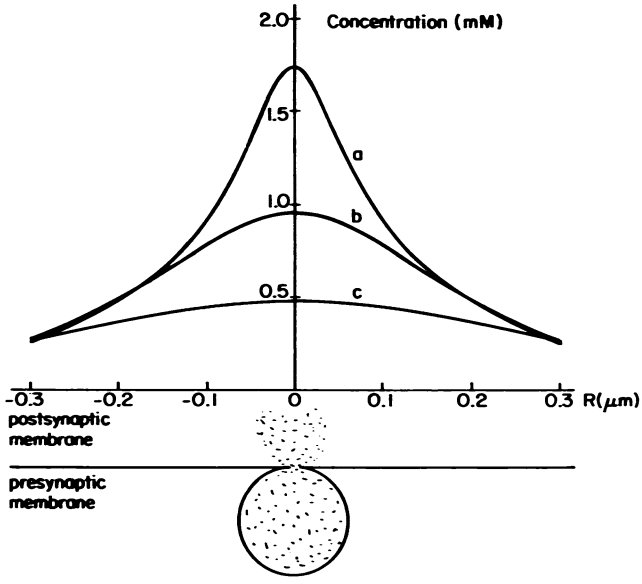

FIGURE 4 Same as in Fig. 3 for a point source of duration $\theta = 100$ μs.

The approximate calculations we have made yield analytic formulas that provide insight into the interaction of the various processes. The price is a degree of inaccuracy. To obtain more accurate results concerning the time of discharge, we performed numerical calculations with FIDAP. The geometrical setup is depicted in Fig. 5 *A*. Initially, the transmitter is confined within a spherical vesicle pore. At $t = 0$ the vesicle opens and transmitter diffuses out through the pore into a large cleft, which models the gap all along the critical zone. Zero concentration boundary conditions were taken at the end of the cleft, but these conditions matter little because

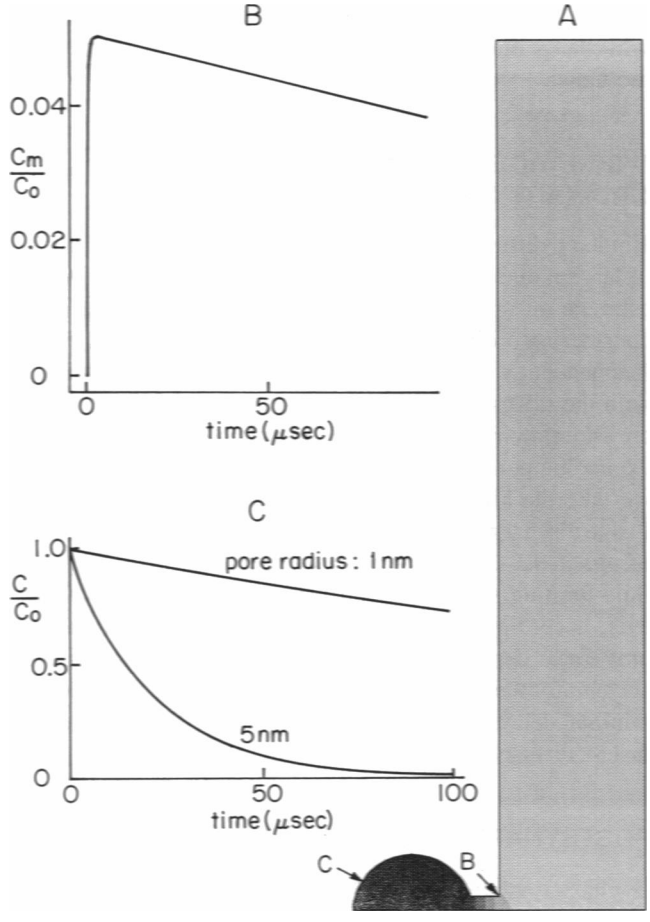

FIGURE 5 Results of FIDAP simulations, with parameters as in Fig. 1. (*A*) Snapshot at $t = 500$ μs after the vesicle opens. Half of the symmetric region is shown, with the vesicle, the pore, and the cleft into which the transmitter diffuses. The gray scale (which contains some artifactual fluctuations not present in the original color output) shows that the concentration ranges from about 8% of C_0 (the initial concentration) in the vesicle to at most 1% in the cleft. (*B*) Concentration C_m at the pore mouth, averaged over the cross-section. (*C*) Average concentrations in the vesicle for two different pore radii. The smaller radius (1 nm) is the best available estimate for the actual vesicular radius (the same as for mast cells). The unrealistically large radius (5 nm) would be required to obtain discharge in <100 μs, as estimated above.

the concentration at the cleft ends is very low throughout the simulated period.

In Fig. 5 *B* we present a graph of the concentration at the pore mouth using the best available biophysical parameters, as in Figs. 1 and 2. We see that the concentration is about 5% of C_0, the initial concentration of neurotransmitter. This nonzero concentration increases the discharge time from the approximate lower bound of 530 μs (expanding pore), shown in Fig. 2, to a value of about 900 μs (fixed pore of initial radius). This is significantly longer than the minimal delay, so that the inappropriateness of diffusive release is even more strongly reinforced.

We will now discuss the role of possible inaccuracies in key parameters. It might be argued that the diffusion coefficient of neurotransmitter molecules could acquire a value significantly higher than the one we used ($4 \cdot 10^{-6}$ cm^2/s). This is not likely to be the case, since the reported diffusion coefficient for molecules of similar size and properties as those of the typical neurotransmitter are $\sim 7 \cdot 10^{-6}$ cm^2/s (Gosting, 1956). These values were measured in water and are expected to be somewhat lower in the cleft and the pore, where the environment is not pure water. However, if we take the highest possible diffusion coefficient, that is of small molecules in water (10^{-5} cm^2/s), discharge via ordinary unhindered diffusion will last ~0.24 ms. This is still too long for the cases of minimal delay of 0.2 ms and cannot provide high concentration at the postsynaptic critical area.

The next parameter to consider is the initial size of the pore and the effect of its expansion. We have shown that even if the duration of the discharge lasts 1 ms, which is unacceptably long, the effect of pore expansion during that period would not be significant (Fig. 2). By contrast, in the case of slow releasing systems, such as chromaffin and mast cells, where the discharge from a single granule lasts tens of milliseconds (Chow et al., 1992; Curran and Brodwick, 1991), expansion of the pore is certainly important.

Recall, however, that until now we used the assumption that the fusion pore in fast synapses is similar in size to that of the mast cells. There is no solid data on the true size of the pore in vesicles. In the face of this we used FIDAP to calculate the minimal pore size that can provide an appropriately rapid diffusive discharge. For a diffusive discharge of 70 μs in duration, the average diameter of the pore should be ~10 nm instead of the 2 nm reported for mast cells (Fig. 5 *C*). The existence of such a large pore during the first 100 μs of discharge seems highly improbable. For example, the diameter of an entire structure that includes a calcium channel (and perhaps other units) has been found to be just 9.2 nm (Pumplin et al., 1981). Later, processes such as intercalation of lipids (Spruce et al., 1990) can greatly enlarge the pore. But this is irrelevant to fast discharge, which has already been completed.

SUMMARY AND DISCUSSION

We showed here that, in contrast to the commonly held view, diffusion is not likely to be the mechanism underlying discharge of neurotransmitter in fast synapses. This conclusion was reached mainly because diffusive discharge is too slow. We based our conclusions on several different lines of argument. This was necessary as in each separate argument some assumptions and approximations were used which could cast doubt on the final inference. Taken together, however, the various arguments seem convincing.

If diffusion is not the mechanism, what does govern discharge? Several theories were proposed for slow releasing systems. The process of discharge from large vesicles was suggested to be caused by the action of actin filaments, or actin-like proteins present on the vesicle membrane, or by cytoskeletal elements acting externally upon the bilayer. Another interesting possibility is expansion of the vesicle matrix caused by monovalent ions as was observed in the secretory granule of the beige mouse (Nanavati and Fernandez, 1993; Curran and Brodwick, 1991). A mechanism of electrodiffusive or ion exchange discharge was demonstrated in mast cells (Uvnas et al., 1985), bovine chromaffin granules, and granule-enriched materials (Uvnas and Aborg, 1984a). This last mechanism might be extrapolated to the case of fast quantal discharge from small synaptic vesicles (Uvnas and Aborg, 1984b). Condider acetylcholine, a positively charged neurotransmitter. We suggest that as such a transmitter passes outward through a newly opened pore there is a compensating inward flow of sodium ions. In a future publication we will show that such ion exchange is the essence of a theory that can provide suitably rapid discharge.

Let us consider some more general implications of our findings that discharge must be very fast, in the range of <100 μs. Recall that from the independence of release kinetics on calcium and voltage it was concluded that the steps that control release must be fast (Lustig et al., 1990). The only other necessary steps in release are membrane fusion and pore formation. Thus these steps must be rate-limiting in fast synapses and therefore temperature-dependent. Indeed, it was recently shown that the rate of pore formation in the secretory granules during exocytosis is strongly affected by temperature, with $Q_{10} = 4.1$ (Oberhauser et al., 1992). Because at room temperature the entire process cannot last longer than 0.5 ms in fast synapses, both membrane fusion and pore formation take place in a fraction of a millisecond. If mechanisms of fusion and pore formation are common in fast and slow releasing systems, which is believed to be the case (Monck and Fernandez, 1992, Zimmerberg et al., 1993), then also in slow systems fusion and pore formation take place in <1 ms.

APPENDIX

Let us consider diffusive discharge from the vesicle through a constant pore taking into account volume and surface changes due to the water flow through a permeable vesicle membrane. Water flows through the membrane according to the law

$$q = L_p s(\Delta\pi - \Delta p) = L_p s(RT\Delta C - \Delta p). \quad \text{(A1)}$$

In Eq. A1, R is the universal gas constant, T is the absolute temperature, L_p is the permeability coefficient of the mem-

brane, s is the vesicle membrane surface area, and $\Delta\pi$ and Δp are the differences in osmotic and hydrostatic pressure, respectively. We assume that $\Delta p = 0$ and that the concentration outside the vesicle is fixed at 0.

Suppose that the vesicle surface and the volume, V, change while the vesicle itself remains spherical: $s(V) = \delta V^{2/3}$, $\delta = 3^{2/3}(4\pi)^{1/3}$. The diffusion of substance from the vesicle of varying volume can be approximated by two equations

$$\frac{dN}{dt} = -\frac{DS}{L}\frac{N}{V}, \qquad \frac{dV}{dt} = L_p \delta RT \frac{N}{V^{1/3}}. \tag{A2}$$

Here $N(t) = C(t)V(t)$. The time of discharge is determined by

$$t \sim \tau(1 + \alpha\tau)\ln\frac{C}{C_0}, \qquad \alpha = L_p \delta RT \frac{N_0}{V_0^{4/3}}, \qquad \tau = \frac{V_0 L}{\pi r_0^2 D}. \tag{A3}$$

With the parameters from neuromuscular junction (Figs. 1 and 2) and a typical value of permeability coefficient of the vesicle membrane, $5 \cdot 10^{-12}$ cm^3/(dyn·s), the time required for the discharge of 95% of the vesicle contents via ordinary diffusion is 0.61 ms in comparison with 0.6 ms calculated by Eq. 3 with $b = 0$. Thus, vesicle volume and surface changes will not affect the estimates for the time of the diffusive discharge.

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